

## Pressure Overload-induced Hypertrophy in Transgenic Mice Selectively Overexpressing AT2 Receptors in Ventricular Myocytes.

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## Abstract

*Background:* The role of the Angiotensin II type 2 receptor (AT2) in cardiac hypertrophy remains controversial. Here we studied the effects of AT2 on chronic pressure overload-induced cardiac hypertrophy in transgenic mice selectively overexpressing AT2 receptors in ventricular myocytes.

*Methods:* Left ventricular hypertrophy was induced by banding of the ascending aorta (AS). Transgenic mice overexpressing AT2 (AT2TG-AS) and non-transgenic mice (NTG-AS) were studied after 70 days of aortic banding. Non-banded NTG mice were used as controls. Left ventricular (LV) function was determined by catheterization via LV puncture and cardiac magnetic resonance imaging. LV myocyte diameter and interstitial collagen were determined by confocal microscopy. ANP and BNP were analyzed by Northern blot. SERCA2, iNOS, eNOS, ERK1/2, P70S6K, SHP-1 and PP2A were analyzed by Western blot.

*Results:* LV myocyte diameter and collagen were significantly reduced in AT2TG-AS compared to NTG-AS mice. LV anterior and posterior wall thickness were not different between AT2TG-AS and NTG-AS mice. LV systolic and diastolic dimensions were significantly higher in AT2TG-AS than in NTG-AS mice. LV systolic pressure and LV end-diastolic pressure were lower in AT2TG-AS than in NTG-AS mice. ANP and BNP were not different between AT2TG-AS and NTG-AS mice. SERCA2 was not different between AT2TG-AS and NTG-AS mice. PLB and the PLB/ SERCA2 ratio were significantly higher in AT2TG-AS than in NTG-AS mice. iNOS was higher in AT2TG-AS than in NTG-AS mice but not significantly different.

*Conclusion:* Our results indicate that AT2 receptor overexpression modified the pathological hypertrophic response to aortic banding in transgenic mice.

Key Words: Cardiomyocyte, AT2 receptor, Hypertrophy, Gene Expression, Protein Expression

## Introduction

The cardiac effects of the type 1 angiotensin II (AT1) receptor are well established but those of the type 2 angiotensin II (AT2) receptor remain controversial (6, 38, 53). It has been reported that pathological hypertrophy and failure of the human heart resulted in a decrease in AT1 receptor expression and in an increase or no change in AT2 receptor expression (25). These reports are consistent with an increase in the AT2/AT1 receptor ratio and imply a relative increase in angiotensin II (Ang II) signaling via the AT2 receptor in hypertrophied hearts. Therapeutic approaches using angiotensin-converting enzyme (ACE) inhibitors or AT1 receptor antagonists to modify cardiac hypertrophy and failure aim to block AT1 receptor signaling. However, ACE inhibitors reduce the amount of Ang II available to bind to AT2 receptors, whereas AT1 receptor inhibition results in an accumulation of Ang II, which may stimulate AT2 receptors (25).

Studies in cultured coronary endothelial cells, fibroblasts and neonatal cardiac myocytes suggested that AT2 receptor activation inhibits cell growth and proliferation, thus opposing the effects of the AT1 receptor (6, 45, 48). Studies using AT2 antagonists or AT2 receptor knockout mouse models of myocardial infarction have shown that blockade or deletion of the AT2 receptor increased the mortality rate and severity of heart failure (1, 32, 23, 50). Findings in AT2 receptor knockout mice with pressure overload-induced hypertrophy or Ang II infusion-induced hypertrophy demonstrated either a requirement or no effect of the AT2 receptor on cardiac hypertrophy (2, 16, 39). Furthermore, Kurisu et al showed that Ang II infusion in transgenic mice overexpressing the AT2 receptor in cardiomyocytes had no effect on cardiomyocyte hypertrophy (21).

However, others have reported that AT2 receptor gene transfer attenuated cardiac hypertrophy and fibrosis in spontaneously hypertensive rats (11, 26).

Here, we studied whether AT2 receptor overexpression in ventricular cardiomyocytes modifies pressure overload-induced cardiac hypertrophy and function in transgenic mice.

## Methods

### Mouse Model of Cardiac Hypertrophy

Left ventricular hypertrophy was induced by banding the ascending aorta in male transgenic mice (AT2TG-AS, n=42) in which ventricular myocyte-specific overexpression of the AT2 receptor was driven by the myosin light chain 2v promoter (copy number = 9) and in non-transgenic male littermates (NTG-AS, n=45) (age: 4.5 weeks and weight: 19-22 g for both groups). We have previously demonstrated that these transgenic mice and age-matched NTG littermates showed no difference in survival, histology and LV function (51). Age-matched male NTG littermates that did not undergo aortic banding were used as controls (n=26). A separate cohort of AT2TG-AS and NTG-AS mice was studied after 10 days of aortic banding to confirm that the degree of LV hypertrophy was comparable in both groups.

### Cardiomyocyte Diameter and Cardiac Collagen Content

LV myocyte transverse diameter was determined by confocal microscopy in controls (n=120 myocytes from 10 mice), NTG-AS (n=72 myocytes from 6 mice) and AT2TG-AS mice (n=84 myocytes from 7 mice) as previously described (10). Animals were studied ~ 70 days after aortic banding (NTG-AS mice:  $76 \pm 7$  days and AT2TG-AS mice:  $72 \pm 2$  days). In brief, hearts were removed and rinsed in 0.1 mol/L phosphate buffer solution (PBS) with 50 mmol/L KCl (pH 7.2) and subsequently fixed overnight at 4° C in 4% paraformaldehyde prepared in PBS. Vibratome sections (100  $\mu$ m) of comparable areas of the LV free wall and the septum were stained with a 1:20 dilution of rhodamine phalloidin (Invitrogen, Carlsbad, CA) and imaged with a BioRad MRC 1000 confocal scanning laser microscope. A minimum of 5 optical sections were obtained from the

free wall of the LV of each animal using a Nikon x60 (NA 1.4) objective. Myocyte diameters were measured perpendicular to the long axis of the sarcomeres from unbranched areas of the myocytes near an intercalated disk using the length/profile function (BioRAD MRC-1000 COMOS software).

LV collagen content was measured in paraffin-embedded tissue sections from the median part of the heart stained with Mallory's Trichrome resulting in blue stained collagen and red stained remaining cardiac tissue. The blue stained interstitial collagen was determined in controls (n=27 from 3 hearts), NTG-AS (n=45 from 5 hearts) and AT2TG-AS mice (n=27 from 3 hearts). Ten (x40) microscopic fields from each section were randomly imaged and the interstitial collagen volume fraction determined as previously described by Robert et al (34). Image analysis was performed using MetaMorph 6.1 image analysis software (Molecular Devices Corp, Downingtown, PA). Blue stained collagen was chosen as the Inclusive (I) region of interest and the remaining red stained cardiac tissue was chosen as the Excluded (E) region. Collagen content between groups was determined as  $I/E \times 100$  and statistics were performed using Sigma Stat Software (SPSS, Inc, Chicago, IL).

### **LV Function**

LV function was measured by catheterization via LV puncture, cardiac magnetic resonance imaging (MRI) after 70 days of aortic banding; and echocardiography after 10 days of aortic banding. Cardiac MRI was performed using a 4.7 Tesla microimaging system (Biospec™, Bruker BioSpin MRI, Inc., Karlsruhe, Germany). Mice were anesthetized by inhalation of 1-2% isoflurane (IsoFlo®, Abbott Laboratories, North Chicago, IL) and were placed prone with electrodes for cardiac gating and a respiratory

sensor. Low-resolution multi-slice images, serving as the end-expiratory phase localizer, were first acquired to obtain the orientation of the heart using a fast spin echo sequence. A left ventricular short axis slice at the level of the papillary muscles was acquired using a gradient echo sequence with cardiac and respiratory triggering. A repetition time of 1/10 of the duration of a single cardiac cycle (180-210 msec per beat) was selected to obtain 10 images per cardiac cycle. Other scan parameters were: matrix  $128 \times 128$ , minimum effective echo time 1.8–2.1 msec, field of view 2.5–3 cm, slice thickness 1 mm, and number of excitation 4, resulting in a total scan time of ~ 6 minutes. The following parameters were determined: posterior and anterior wall thickness, relative wall thickness, systolic and end-diastolic diameter and endocardial fractional shortening.

### **Northern Blot Analysis**

LV mRNA levels of atrial natriuretic polypeptide (ANP) and brain natriuretic peptide (BNP) were analyzed by Northern blot (plasmids kindly provided by Dr. Julie McMullen) in controls (n=6), NTG-AS (n=3) and AT2TG-AS mice (n=3) as previously described (41). In brief, mRNA was purified from LV tissues using TRI Reagent (Sigma). An aliquot of 20  $\mu$ g of RNA was separated in a 1.5% of denaturing formaldehyde agarose gel and transferred to a Hybond N (Ambion, Austin, TX) membrane. The blots were then hybridized with ANP, BNP or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes at 42° C in a hybridization buffer (50% deionized formamide, 6x SSC, 5xDenhardt's solution, 0.5% SDS, 200 $\mu$ g/ml denatured salmon sperm DNA). Blots were washed twice at room temperature in 2 x SSC-1% SDS and then twice at 50°C in 2 x SSC for 30 minutes. The signals were visualized by autoradiography and quantified by ImageQuant software (Molecular Dynamics,



Sunnyvale, CA). Densitometric values of ANP and BNP mRNA levels were normalized by GAPDH mRNA levels.

### **Western Blot Analysis**

LV protein levels were analyzed by Western blot with specific antibodies in controls (n=6) , NTG-AS (n=5) and AT2TG-AS mice (n=5): phospholamban (PLB), sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase 2 (SERCA2) (Affinity Bioreagents, Golden, CO), inducible nitric oxide synthase (iNOS), phosphorylated and total endothelial nitric oxide synthase (eNOS) (BD Biosciences, Franklin Lakes, NJ), phosphorylated and total ERK1/2, p70 S6 Kinase (p70S6K), (Cell Signaling Technology, Danvers, MA), Src-homology 2 domain-containing protein tyrosine phosphatase (SHP-1) (Abcam, Cambridge, MA) and protein serine/threonine phosphatase 2A (PP2A) (Cell Signaling Technology, Danvers, MA). GAPDH was used as a loading control.

### **Statistical Analysis**

Statistical analysis was performed using ANOVA and Tukey's post hoc test (SigmaStat). Differences were considered significant with  $P < 0.05$ . Kaplan-Meier estimates of survival were determined using SAS for Windows v6.12 (SAS Institute, Cary, NC). Results are presented as mean  $\pm$  SEM.

## Results

### Survival

Survival was not significantly different in AT2TG-AS mice (81%) compared to NTG-AS mice (70%) after 70 days of aortic banding. There was a trend of increased mortality in NTG-AS mice relative to AT2TG-AS mice during the first 14 days following surgery, suggesting improved early survival in AT2-AS mice. There was, however, no difference in late survival between AT2TG-AS and NTG-AS mice (Figure 1).

### Cardiomyocyte Diameter and Cardiac Collagen Content

We measured LV cardiomyocyte transverse diameters and LV interstitial collagen content in AT2TG-AS, NTG-AS and control mice to determine the extent to which AT2 receptor overexpression affects LV hypertrophy after 70 days of aortic banding. Cardiomyocyte transverse diameters were significantly reduced in AT2TG-AS ( $15.1 \pm 0.7 \mu\text{m}$ ,  $n=84$  myocytes from 7 mice) compared to NTG-AS mice ( $17.1 \pm 0.4 \mu\text{m}$ ,  $n=72$  myocytes from 6 mice,  $P<0.05$ ) but was significantly higher in both groups compared to control mice ( $12.6 \pm 0.3 \mu\text{m}$ ,  $n=120$  myocytes from 10 mice,  $P<0.05$ ). LV interstitial collagen content was significantly reduced in AT2TG-AS ( $15.2 \pm 0.1 \%$ ,  $n=27$  areas per heart from 3 mice) compared to NTG-AS mice ( $26.0 \pm 0.2 \%$ ,  $n=45$  areas per heart from 5 mice,  $P<0.05$ ) but was significantly higher in both groups compared to control mice ( $6.0 \pm 0.03 \%$ ,  $n=27$  areas per heart from 3 mice,  $P<0.05$ ) after 70 days of aortic banding (Figure 2). LV weight (LVW) and the LVW/body weight (LVW/BW) ratio were significantly increased in AT2TG-AS and NTG-AS mice compared to control mice but not different between AT2TG-AS and NTG-AS mice. LV anterior, posterior wall

thickness and relative wall thickness were not different between AT2TG-AS and NTG-AS mice (Table 1).

We determined the extent of cardiac hypertrophy in AT2TG-AS, NTG-AS and control mice after 10 day of aortic banding to further confirm that the initial degree of pressure overload was similar in both groups. LVW was significantly increased in AT2TG-AS and NTG-AS mice compared to age-matched control mice but not different between AT2TG-AS and NTG-AS mice. LVW/BW ratio was significantly lower in AT2TG-AS compared to NTG-AS mice. The difference in LVW/BW ratio between AT2TG-AS and NTG-AS mice after 10 days was possibly attributable to slight variations in body weight during the recovery period after surgery in the young animals as LVW did not differ between both groups. MRI measurements were not performed in the young small animals. Echocardiography showed a significant increase in posterior, anterior and relative wall thickness in AT2TG-AS and NTG-AS mice compared to control mice but these indices were not different between AT2TG-AS and NTG-AS mice. These data indicate that both the magnitude of pressure overload and hypertrophy were similar between AT2TG-AS and NTG-AS mice after 10 days of aortic banding (Table 2).

### **LV Function**

We performed cardiac catheterization via LV puncture and MRI measurements to determine LV function. LV systolic pressure was significantly lower in AT2TG-AS compared to NTG-AS mice. LV end-diastolic pressure was slightly lower in AT2TG-AS compared to NTG-AS mice. LV systolic pressure per gram of LV mass (LV developed pressure), an index of force development per unit of myocardium, was significantly lower in AT2TG-AS compared to NTG-AS mice. LV positive dP/dt, and negative dP/dt

were significantly reduced in AT2TG-AS compared to NTG-AS mice. LV systolic and diastolic dimensions were significantly higher in AT2TG-AS compared to NTG-AS mice, and LV endocardial fractional shortening was lower in AT2TG-AS compared to NTG-AS mice. These findings indicate a modified LV function in AT2 receptor overexpressing mice compared to non-transgenic mice with pressure overload-induced hypertrophy (Table 1).

We performed hemodynamic measurements 10 days after aortic banding in a separate cohort of mice to confirm that AT2TG-AS and NTG-AS mice had a similar degree of initial pressure overload compared to controls. There were no significant differences in LV peak systolic pressure, LV end-diastolic pressure, LV developed pressure, peak +dP/dt and peak -dP/dt between AT2TG-AS and NTG-AS mice after 10 days of aortic banding (Table 2).

### **Northern Blot Analysis**

We determined LV mRNA expression of ANP and BNP, which is prototypical of hypertrophy, in AT2TG-AS and NTG-AS mice. ANP and BNP were significantly higher in AT2TG-AS and NTG-AS compared to control mice after 70 days of aortic banding but there was no difference in ANP and BNP between AT2TG-AS and NTG-AS mice (Figure 3).

ANP and BNP were also increased in AT2TG-AS and NTG-AS compared to control mice after 10 days of aortic banding but there was no difference in ANP and BNP between AT2TG-AS and NTG-AS mice (ANP:  $1186 \pm 245\%$  and  $1183 \pm 273\%$  versus  $100 \pm 20\%$ ,  $P < 0.05$ , respectively; BNP:  $481 \pm 82\%$  and  $332 \pm 67\%$  versus  $100 \pm 13\%$ ,  $P < 0.05$ , respectively).

## Western Blot Analysis

We measured LV protein expression of PLB and SERCA2 after 70 days of aortic banding to determine mechanisms contributing to changes in LV function in AT2TG-AS mice. PLB was significantly higher in AT2TG-AS and NTG-AS compared to control mice but was significantly higher in AT2TG-AS than in NTG-AS mice. SERCA2 was not different between the groups. The PLB/SERCA2 ratio was significantly higher in AT2TG-AS and NTG-AS compared to control mice but was significantly higher in AT2TG-AS than in NTG-AS mice (Figure 4).

We measured LV protein expression of phosphorylated eNOS, total eNOS and iNOS to determine whether changes in cardiac function are related to an increase in NO production in AT2TG-AS mice. There was no difference between AT2TG-AS, NTG-AS and control mice in phosphorylated eNOS (AT2TG-AS:  $102 \pm 13$  %, NTG-AS:  $89 \pm 4$  %, control mice:  $100 \pm 15$  %, NS) and in total eNOS (AT2TG-AS:  $132 \pm 6$  %, NTG-AS:  $100 \pm 9$  %, control mice:  $100 \pm 9$  %, NS). iNOS was not different between AT2TG-AS and NTG-AS mice but significantly higher in AT2TG-AS than in control mice (Figure 5).

Phosphorylated P70S6K was not different between AT2TG-AS and NTG-AS hearts but significantly higher in AT2TG-AS and NTG-AS compared to control mice (Figure 6).

Phosphorylated and total ERK1/2 were also not significantly different between AT2TG-AS and NTG-AS mice (data not shown).

Studies in cultured neuronal cells have shown that activation of protein phosphatases, such as PP2A and SHP-1, may be related to anti-growth and anti-proliferation effects of the AT2 receptor (5, 15). Therefore, we measured PP2A and SHP-1. There was no difference between AT2TG-AS, NTG-AS and control mice in PP2A (AT2TG-AS:  $92 \pm 20$  %, NTG-AS:  $105 \pm 10$  %, control mice:  $100 \pm 20$  %, NS) and SHP-1 (AT2TG-AS:  $88 \pm 5$  %, NTG-AS:  $100 \pm 12$  %, control mice:  $100 \pm 12$  %, NS).

## Discussion

Our results indicate that AT2 receptor overexpression modified the pathological response to chronic pressure overload in transgenic mice: (1) cardiomyocyte diameter and interstitial collagen were reduced, (2) cardiac function was modified, (3) hypertrophy-related gene and protein expressions were altered and (4) survival was not affected.

### Cardiac Hypertrophy

The role of the AT2 receptor in cardiac hypertrophy is controversial. It has been shown in isolated rat cardiomyocytes that overexpression of the AT2 receptor with different ratios of AT2 and AT1 receptors does not prevent AT1 receptor-mediated myocyte hypertrophy (7). However, others have shown in neonatal rat cardiac myocytes that AT2 receptor stimulation inhibits the growth of cardiomyocytes and cardiac fibroblasts by counteracting AT1 receptor signaling (6, 48). Studies in mice with genetic deletion of the AT2 receptor and pressure overload-induced hypertrophy and in models of Ang II-induced cardiac hypertrophy have demonstrated that the AT2 receptor is either required for cardiac hypertrophic growth or that the receptor has no effects on myocyte hypertrophy (2, 16, 39). Further studies have shown that neither AT2 receptor deletion nor overexpression affects cardiac hypertrophy in transgenic mice (2, 21). In contrast, studies using AT2 receptor gene transfer in spontaneously hypertensive rats have demonstrated a decrease in cardiac hypertrophy and a reduction in myocardial fibrosis (11, 26). In our study, the LV collagen content was significantly reduced and, additionally, the cardiomyocyte diameter was significantly smaller in AT2TG-AS compared to NTG-AS mice (Figure 2). We found, however, that the heart weight and

heart weight/body weight ratio, as well as the LV anterior and posterior wall thickness were not different between AT2TG-AS and NTG-AS mice suggesting an increase in myocyte length rather than width in AT2TG-AS compared to NTG-AS hearts. This assumption is supported by our previous study demonstrating that myocyte length was significantly greater in transgenic mice with high level AT2 receptor overexpression compared to non-transgenic mice (31). Myocytes account for only one-third of the number of cells but their volume accounts for over two-thirds of the myocardium. Assuming that the myocyte volume is similar in AT2TG-AS and NTG-AS mice, the LVW and LV/BW ratio would remain the same in AT2TG-AS and NTG-AS mice despite thinner myocytes and less interstitial fibrosis in AT2TG-AS mice indicating a change in myocyte length.

It has been shown that the AT2 receptor affects the expression of genes and protein in cardiac hypertrophy. ANP and BNP regulate cellular growth, cellular proliferation and cardiac hypertrophy (27, 42, 46). Both ANP and BNP oppose the hypertrophic effect of Ang II and aldosterone on cardiomyocytes (28, 35). SHP-1 expression has been shown to exert a negative regulating effect on cellular proliferation (44) and increased PP2A activity was accompanied by cardiac hypertrophy (12). P70S6K plays also an important role in cardiac hypertrophy (13, 36, 37). For example, Ha et al showed that pressure overload-induced hypertrophy was associated with increased expression of phosphorylated p70S6K (13). Therefore, we measured ANP, BNP, SHP-1, PP2A and p70S6K expression to determine whether AT2 overexpression changed the expression of these molecules. We found that the increase in ANP and BNP expression, typical markers of cardiac hypertrophy, was similar in AT2TG-AS and NTG-AS mice (Figure 3). SHP-1 and PP2A

expression was not increased in AT2TG-AS mice. The increase in phosphorylated p70S6K was not different between AT2TG-AS and NTG-AS mice (Figure 6).

### **LV function**

AT2 receptor expression has been associated with beneficial cardiovascular effects. Studies using animal models of AT2 receptor gene transfer or AT2 receptor overexpression have demonstrated an improvement in cardiac function. Falcon et al using telemetry showed that the Ang II-induced increase in mean blood pressure was not affected in AT2 receptor transduced rats (11). Yang et al reported that cardiac AT2 receptor overexpression improved left ventricular function at baseline and preserved left ventricular function during post-myocardial infarction remodeling in transgenic mice (52). These findings are in agreement with our observations. AT2TG-AS mice had a significantly lower LV systolic pressure and lower LV end-diastolic pressure than NTG-AS mice. Peak positive dP/dt was significantly lower in AT2TG-AS than in NTG-AS mice. However, dP/dt as an index to assess left ventricular contractile function has limitations. It has been shown in animals and man that dP/dt is dependent on afterload and preload. An increase or decrease in afterload as well as in preload leads to an increase or decrease in dP/dt (24, 49). The lower LV function in AT2TG-AS compared to NTG-AS seems not to be caused by dilated cardiomyopathy and early heart failure in the transgenic mice because the anterior, posterior and relative wall thickness were not different between AT2TG-AS and NTG-AS mice. Given that AT2 overexpression was associated with an increase in diastolic dimension and a slight decrease in end-diastolic pressure in AT2TG-AS mice, it is likely that diastolic compliance was improved in these mice.



Expression of cardiac proteins involved in calcium handling such as iNOS, eNOS and PLB have been associated with alterations in cardiac function (18, 29, 30). The contribution of iNOS and sustained NO production in the development of heart failure, however, is a subject of intense debate (3, 6, 23). Mungrue et al showed that cardiac-specific upregulation of iNOS in transgenic mice resulted in contractile dysfunction (29). However, Heger et al reported that cardiac specific overexpression of iNOS in transgenic mice overexpressing iNOS was not associated with deleterious effects on cardiac hemodynamics (14). Overexpression of the eNOS gene within the vascular endothelium in transgenic mice attenuated cardiac dysfunction (19). Ablation of PLB expression in knockout mice did not improve cardiac function or chamber dilation in a heart failure model (18). In contrast, it has been shown that SERCA2 expression and the PLB/SERCA2 ratio were decreased in patients with severe hypertrophic cardiomyopathy and impaired LV contractile reserve (43) Kiss et al reported in a guinea pig model of pressure overload-induced cardiac failure a decrease in protein expression of the calcium-cycling proteins SERCA2 and phospholamban (20).

Therefore, we measured cardiac SERCA2, PLB, iNOS and eNOS expression to determine whether changes in cardiac function were associated with changes of the expression of these molecules. SERCA2 was not different between AT2TG-AS, NTG-AS and control mice. PLB and the PLB/SERCA2 ratio, which is a major determinant of sarcoplasmic reticulum function, were significantly higher in AT2TG-AS than in NTG-AS and control mice (Figure 4). This may lead to changes in calcium handling that facilitate the changes in systolic function in AT2TG-AS compared to NTG-AS mice.

In conclusion, our results demonstrate that myocardial fibrosis and myocyte diameter are reduced in AT2TG-AS mice indicating that AT2 receptor overexpression in cardiac

myocytes alters the pathological response to aortic banding-induced ventricular hypertrophy in the transgenic mice.

Our study further demonstrates that AT2 overexpression also ameliorates cardiac function in response to chronic pressure overload in the transgenic mice.

Our findings may have therapeutic implications in the pathophysiology of cardiac hypertrophy in the clinical setting.

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## Figure Legends

### Figure 1.

Survival was analyzed by Kaplan-Meier estimates and the Log-rank test. Survival was not significantly reduced in AT2TG-AS (81%, n=47). Survival was significantly lower in NTG-AS (70 %, n=52) compared to control mice (100 %, n=31;  $P<0.05$ ) after 70 days of aortic banding.

### Figure 2.

LV myocyte diameter and interstitial collagen content were determined by confocal microscopy.

A. Cardiomyocyte diameter was significantly lower in AT2TG-AS mice (84 myocytes from 7 mice) compared to NTG-AS mice (72 myocytes from 6 mice) and was significantly higher in both groups compared to control mice (120 myocytes from 10 mice) after 70 days of aortic banding. Single values and Mean; \* $P<0.05$  versus controls, ‡ $P<0.05$  versus NTG-AS mice.

B. LV interstitial collagen was significantly reduced in AT2TG-AS (27 areas per heart from 3 mice) compared to NTG-AS mice (45 areas per heart from 5 mice) but was significantly higher in both groups compared to control mice (27 areas per heart from 3 mice) after 70 days of aortic banding. Mean  $\pm$  SEM; \* $P<0.05$  versus controls, ‡ $P<0.05$  versus NTG-AS mice.

**Figure 3.**

- A. Representative Northern blot of ANP and BNP mRNA expression.
- B. ANP and BNP were significantly increased in both AT2TG-AS (n=3) and NTG-AS (n=3) compared to control mice (n=6) but there was no difference in ANP and BNP between AT2TG-AS and NTG-AS mice after 70 days of aortic banding.
- MEAN  $\pm$  SEM; \* $P$ <0.05 versus controls.

**Figure 4.**

- A. Representative Western blot of SERCA2 and PLB protein expression.
- B. PLB was significantly increased in AT2TG-AS (n=5) and NTG-AS (n=5) compared to control mice (n=6) and was significantly higher in AT2TG-AS compared to NTG-AS mice. SERCA2 was not different between all groups. The PLB/SERCA2 ratio was significantly increased in AT2TG-AS and NTG-AS compared to control mice and was significantly higher in AT2TG-AS than in NTG-AS mice after 70 days of aortic banding.
- MEAN  $\pm$  SEM; \* $P$ <0.05 versus controls, ‡ $P$ <0.05 versus NTG-AS hearts.

**Figure 5.**

- A. Representative Western blot of iNOS protein expression.
- B. iNOS was not different between AT2TG-AS (n=4) and NTG-AS (n=4) mice but significantly increased in AT2TG-AS compared to control mice (n=5) after 70 days of aortic banding. MEAN  $\pm$  SEM; \* $P$ <0.05 versus controls.

**Figure 6.**

A. Representative Western blots of phosphorylated and total P70S6K protein expression.

B. Phosphorylated P70S6K was not different between AT2TG-AS (n=5) and NTG-AS (n=5) mice but significantly increased in AT2TG-AS and NTG-AS compared to control mice (n=6) after 70 days of aortic banding. MEAN  $\pm$  SEM; \* $P$ <0.05 versus controls.

**Table 1. LV Function in AT2TG-AS and NTG-AS mice after 70 days of banding**

	Controls	NTG-AS	AT2TG-AS
<b>LV Hemodynamics:</b>	(n=10)	(n=12)	(n=16)
Body weight (g)	29.8±1.1	29.3±0.7	29.2±0.5
LV weight (mg)	85±3	130±5*	128±4*
LV/body weight (mg/g)	2.9±0.1	4.4±0.1*	4.4±0.1*
Heart rate (bpm)	475±37	476±31	468±26
LV peak systolic pressure (mmHg)	86±5	191±12*	130±10*‡
LV end-diastolic pressure (mmHg)	4.3±0.5	14.2±3.3*	10.3±2.4*
LV developed pressure (mmHg/g)	1038±51	1489±119*	1066±89*‡
Peak +dP/dt (+mmHg/s)	5915±547	9389±472*	5924±417‡
Peak -dP/dt (-mmHg/s)	4032±485	6348±383*	3702±313‡
<b>Cardiac MRI:</b>	(n=5)	(n=7)	(n=5)
Posterior wall thickness (mm)	0.80±0.16	1.30±0.16*	1.41±0.24*
Anterior wall thickness (mm)	0.77±0.06	0.97±0.11	1.05±0.12
LV diastolic dimension (mm)	3.82±0.06	3.41±0.10	4.07±0.20*‡
LV systolic dimension (mm)	2.17±0.07	1.82±0.04	2.25±0.07*‡
LV fractional shortening (%)	43±1	50±1*	44±4
Relative wall thickness (mm/mm)	0.40±0.09	0.75±0.11*	0.71±0.15

Mean ± SE. \*P<0.05 versus control mice; ‡P<0.05 AT2TG-AS versus NTG-AS mice.

**Table 2. LV Function in AT2TG-AS and NTG-AS mice after 10 days of banding**

	Controls	NTG-AS	AT2TG-AS
<b>LV Hemodynamics:</b>	(n=12)	(n=12)	(n=13)
Body weight (g)	22.0±0.8	20.8±0.3	21.7±0.5
LV weight (mg)	62±2	88±3*	83±2*
LV/body weight (mg/g)	2.8±0.1	4.2±0.1*	3.8±0.1*‡
LV peak systolic pressure (mmHg)	86±3	155±10*	141±10*
LV end-diastolic pressure (mmHg)	4.1±0.5	8.2±2.3	9.5±1.2
LV developed pressure (mmHg/g)	1403±63	1758±114*	1704±124
Peak +dP/dt (+mmHg/s)	5911±251	7342±572	6970±691
Peak -dP/dt (-mmHg/s)	3867±289	5381±318*	4511±556
Heart rate (bpm)	545±25	483±25	457±37
<b>Echocardiography:</b>	(n=6)	(n=7)	(n=10)
Posterior wall thickness (mm)	0.67±0.02	1.08±0.02*	1.01±0.02*
Anterior wall thickness (mm)	0.80±0.03	1.08±0.04*	1.04±0.04*
LV diastolic dimension (mm)	3.26±0.08	3.22±0.11	3.22±0.11
LV systolic dimension (mm)	1.59±0.11	1.73±0.16	1.59±0.16
LV fractional shortening (%)	51.2±2.1	47.0±3.1	51.2±3.1
LV midwall fractional shortening (%)	28.5±1.3	22.7±1.9*	27.5±1.9
Relative wall thickness (mm/mm)	0.41±0.02	0.67±0.03*	0.64±0.03*
Heart rate (beats/min)	479±17	561±25	557±25

Mean ± SE. \**P*<0.05 versus control; ‡*P*<0.05 AT2TG-AS versus NTG-AS mice.



Figures

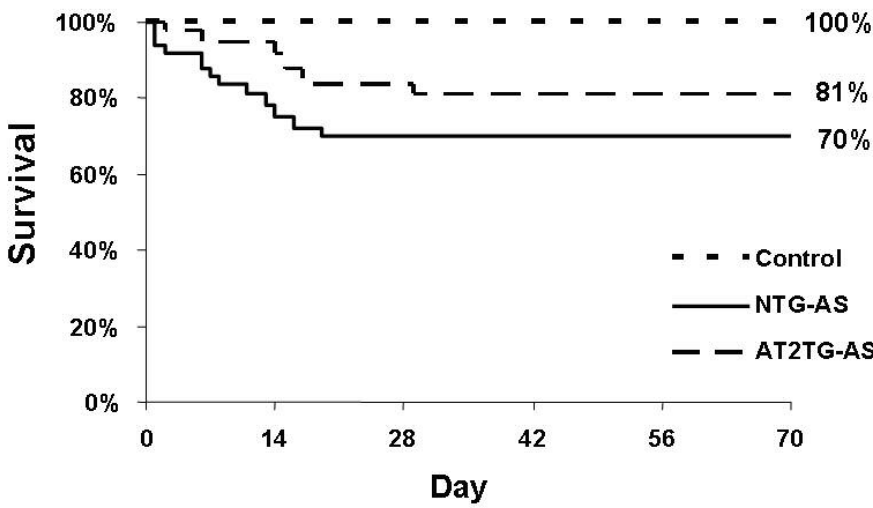


Figure 1

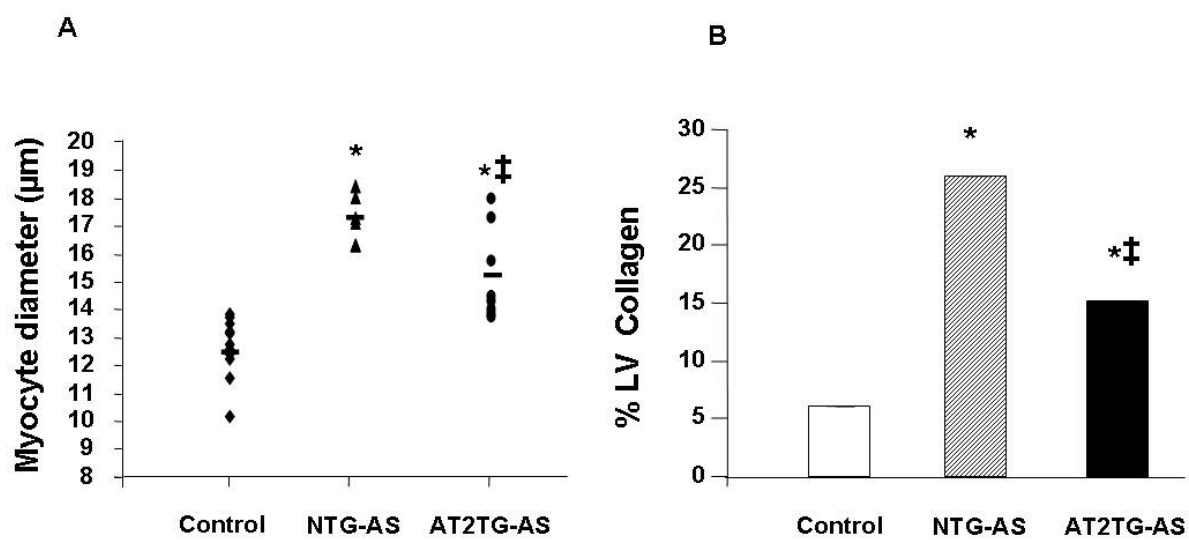


Figure 2

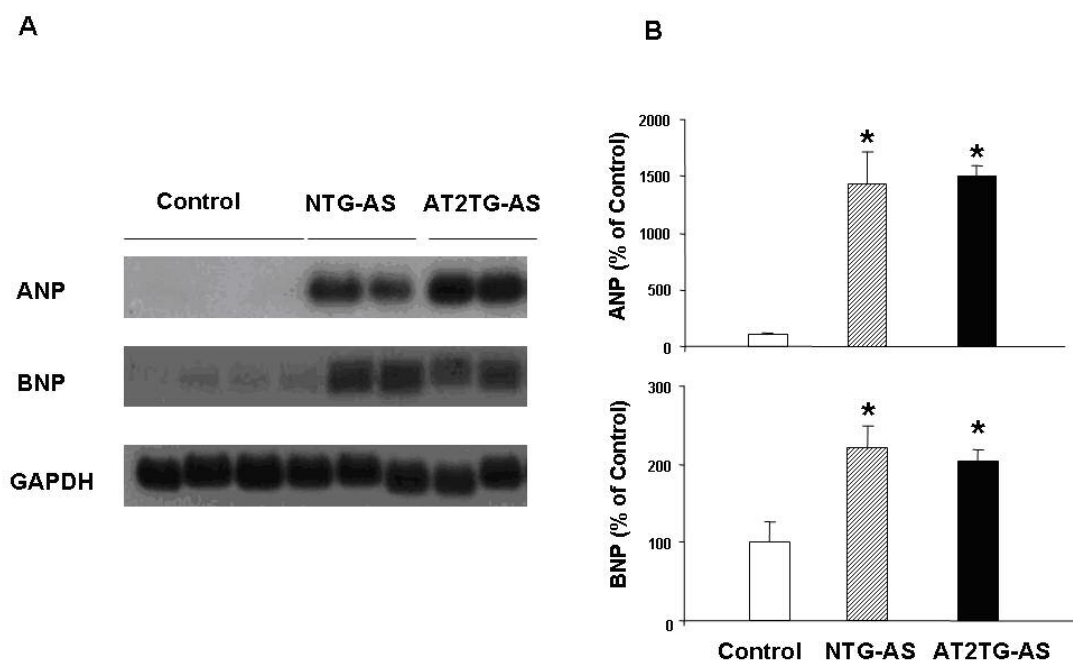


Figure 3

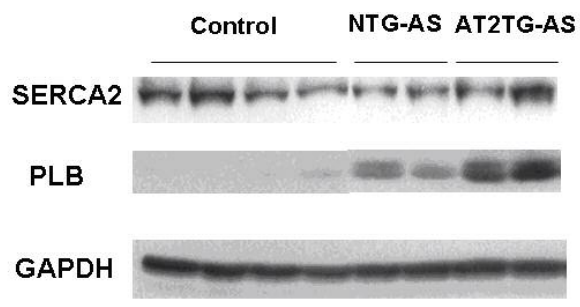


Figure 4A

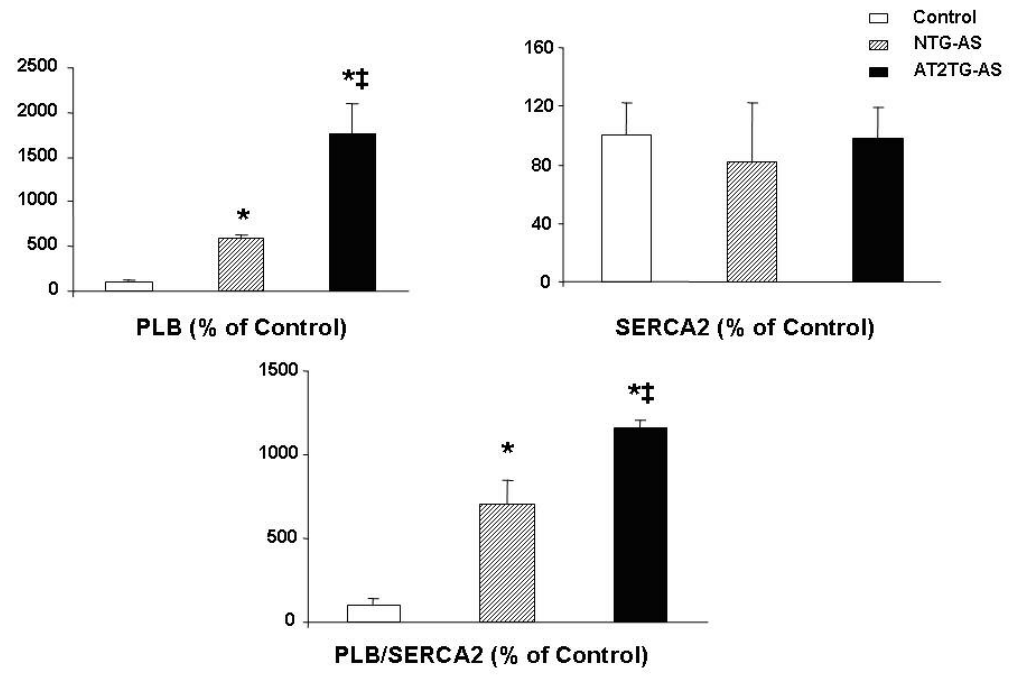


Figure 4B

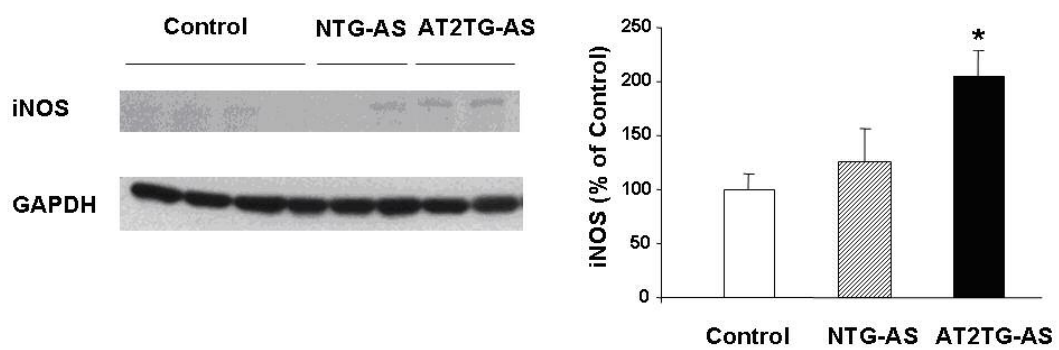


Figure 5

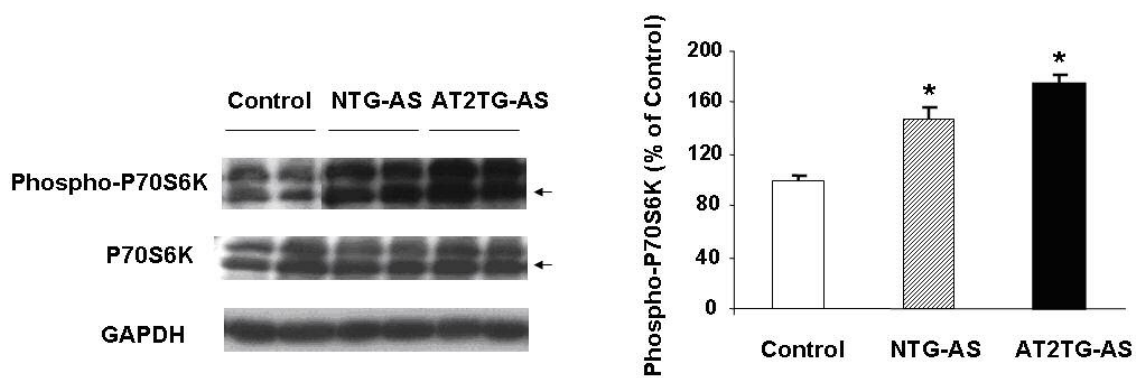


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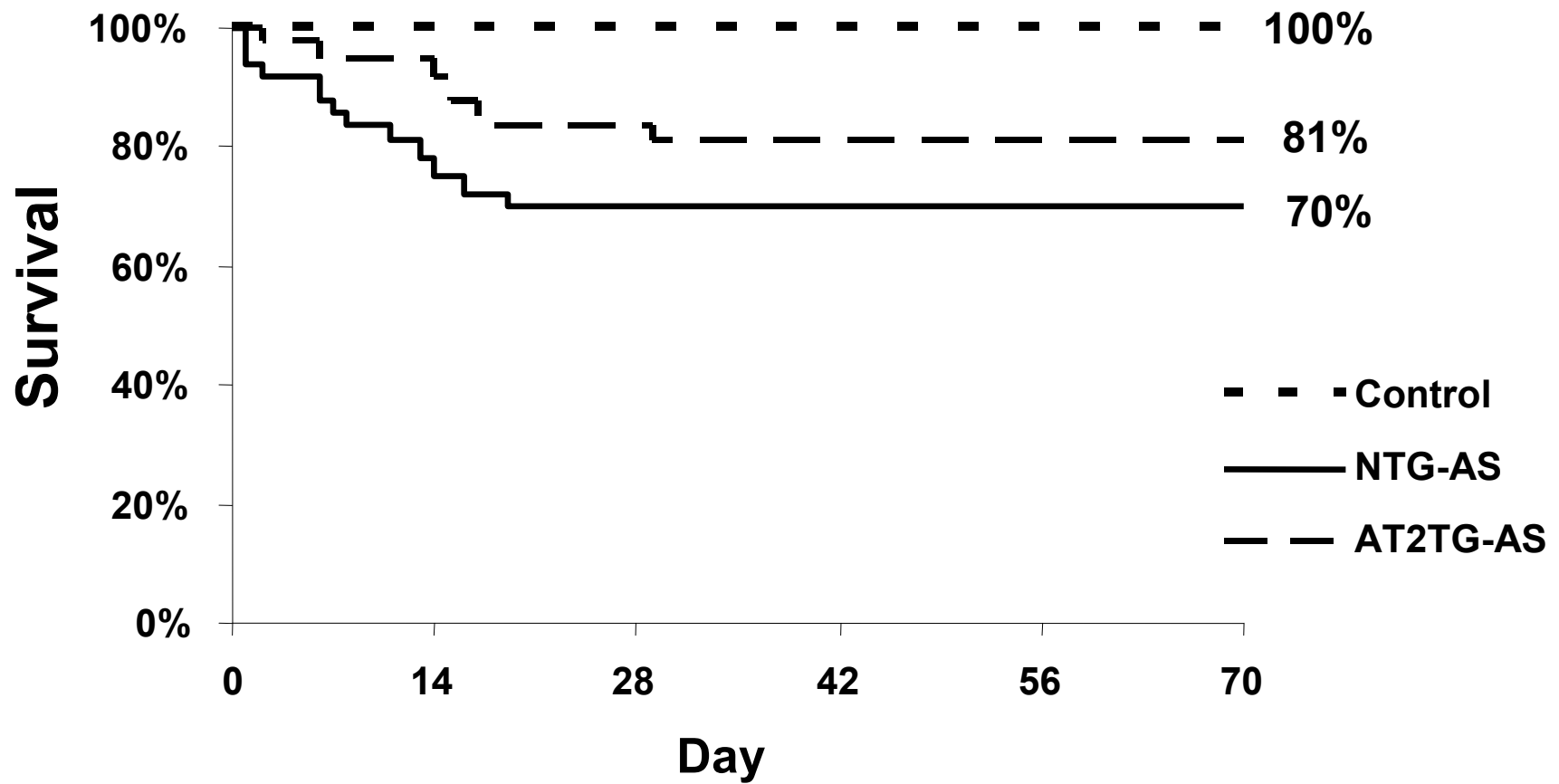


Figure 1

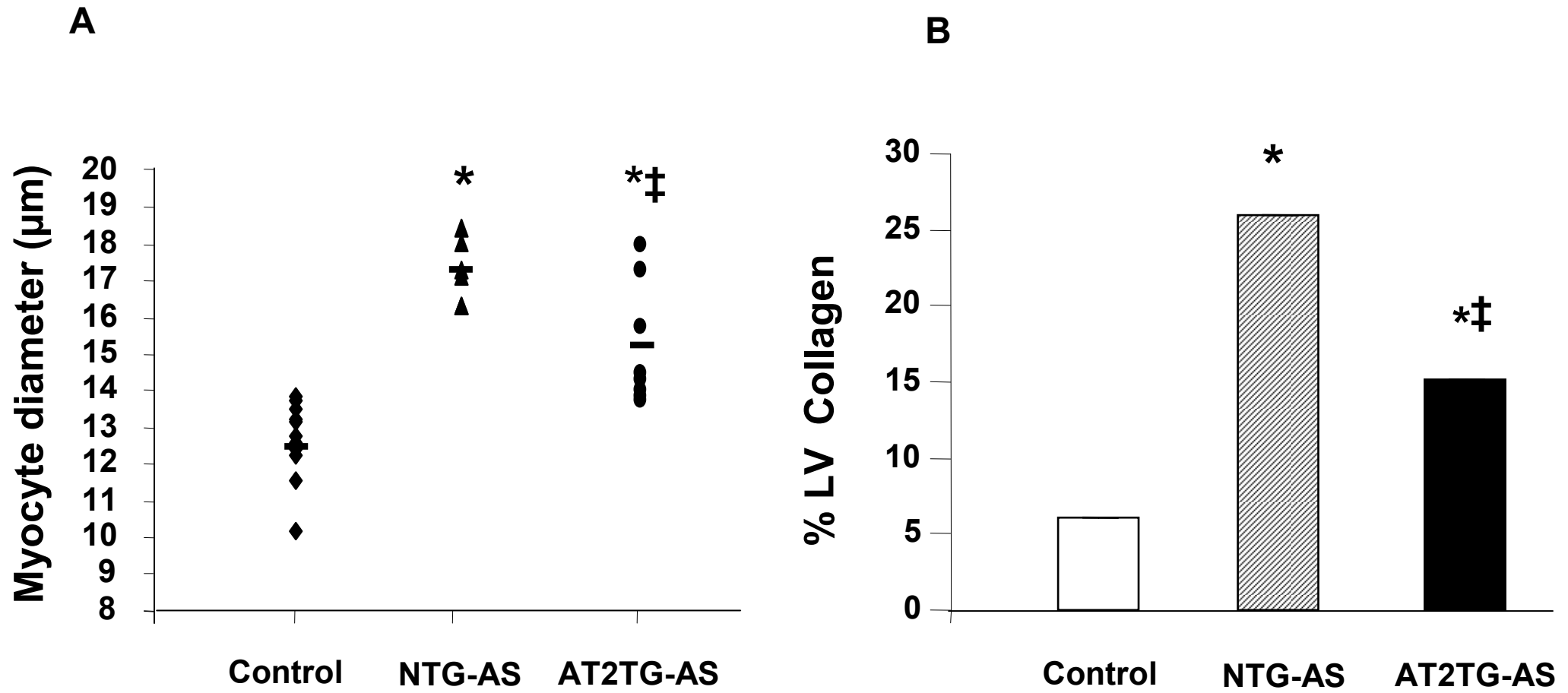
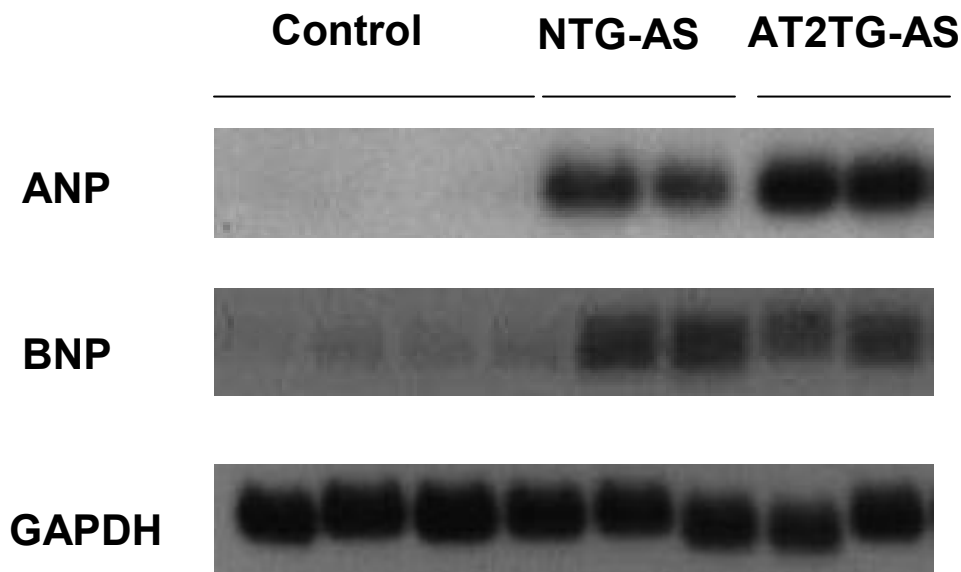
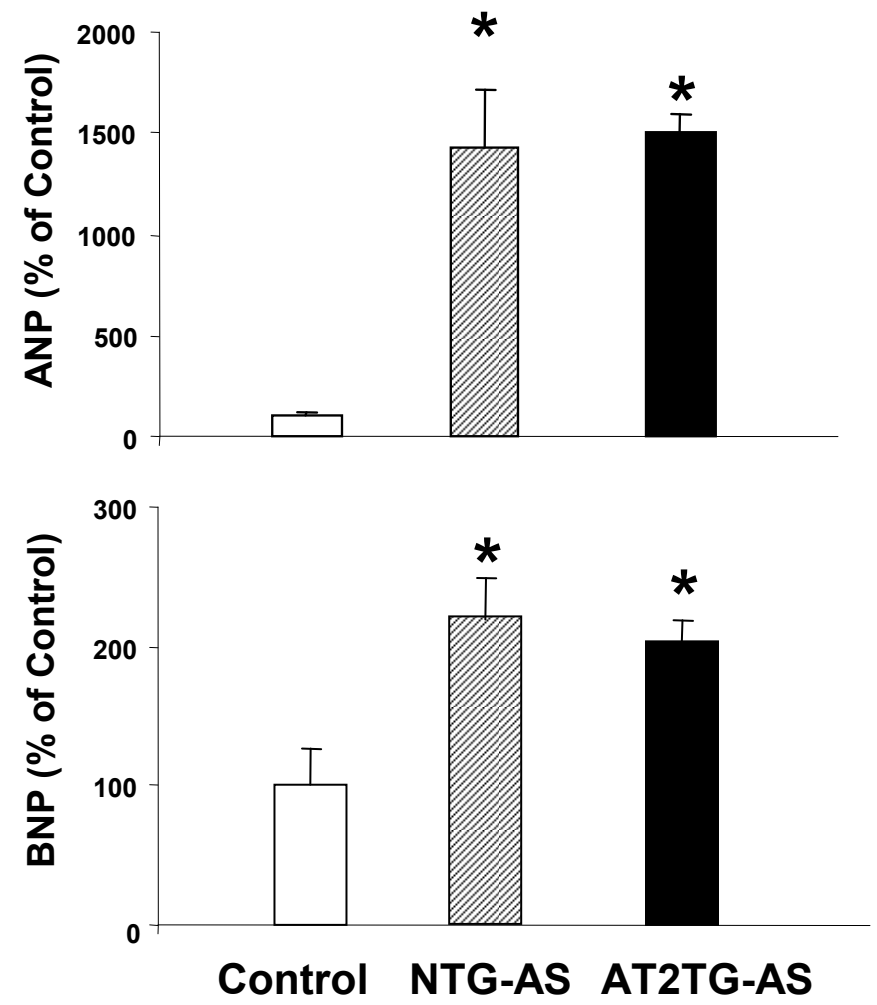


Figure 2

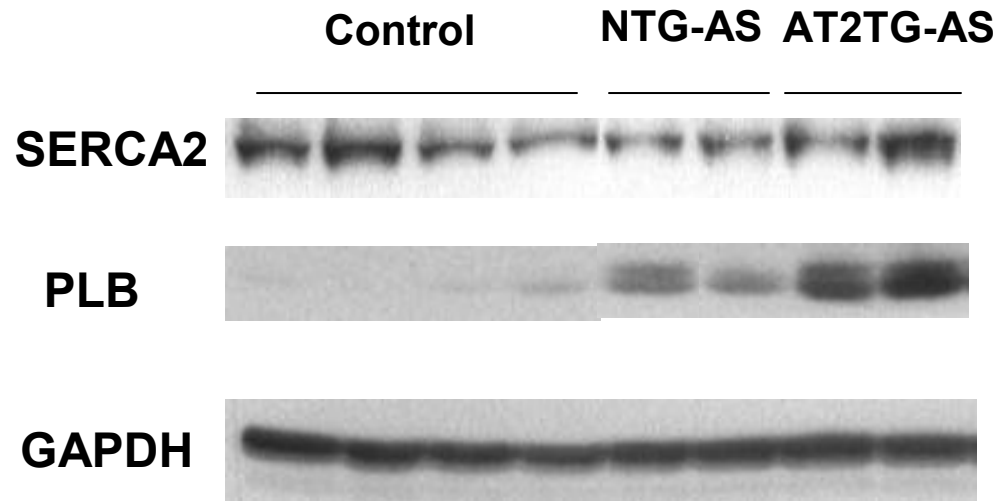
**A**



**B**



**Figure 3**



**Figure 4A**



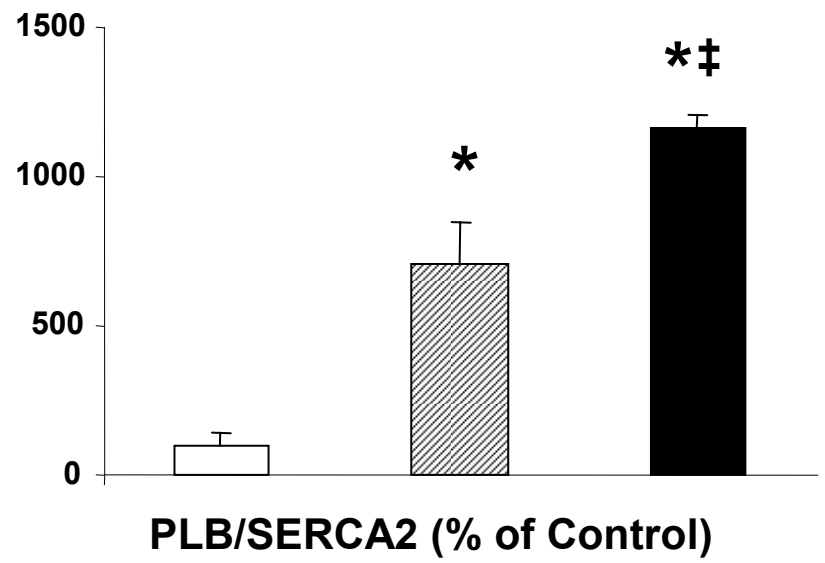
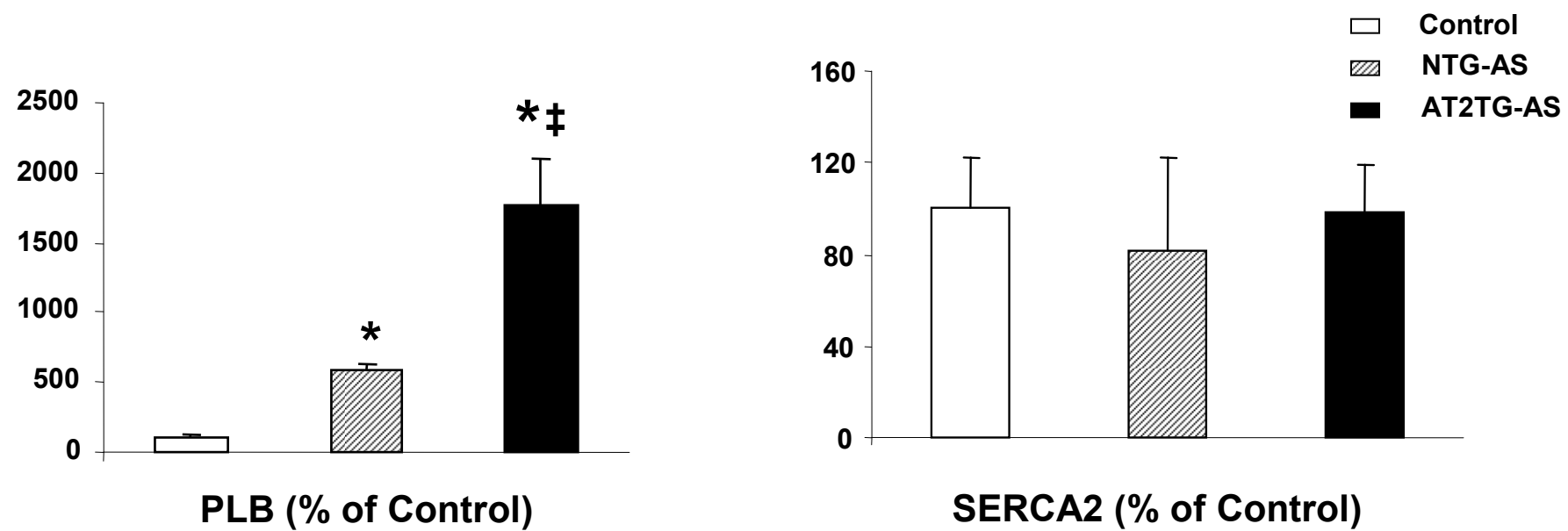


Figure 4B

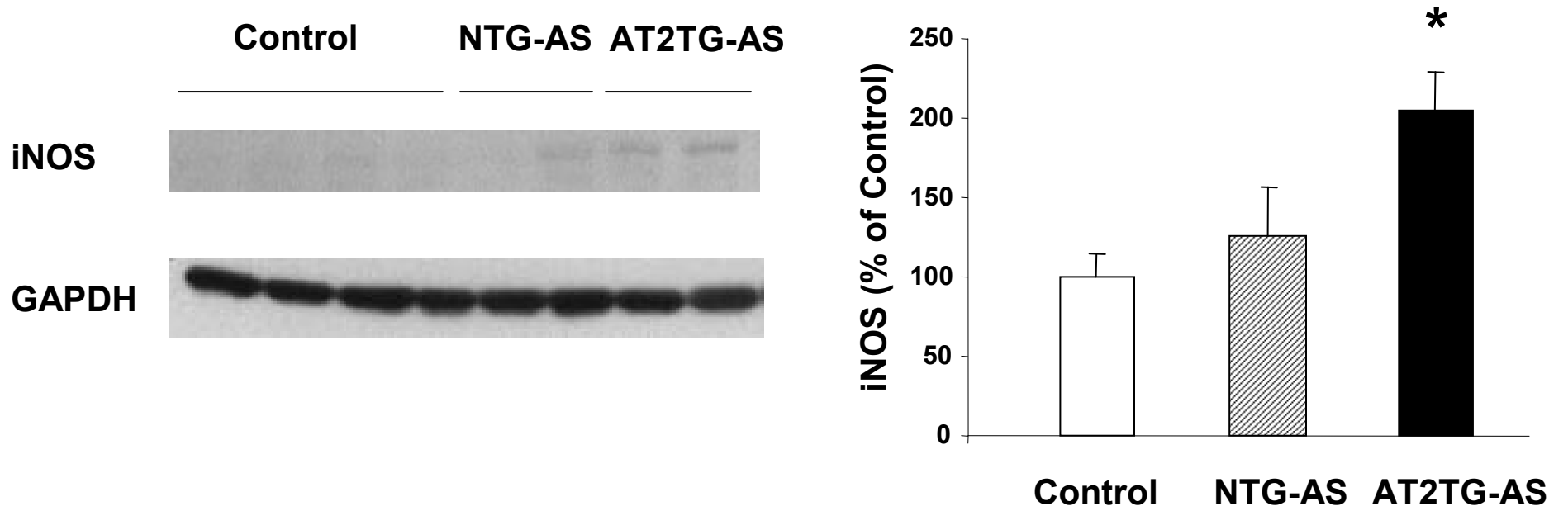


Figure 5

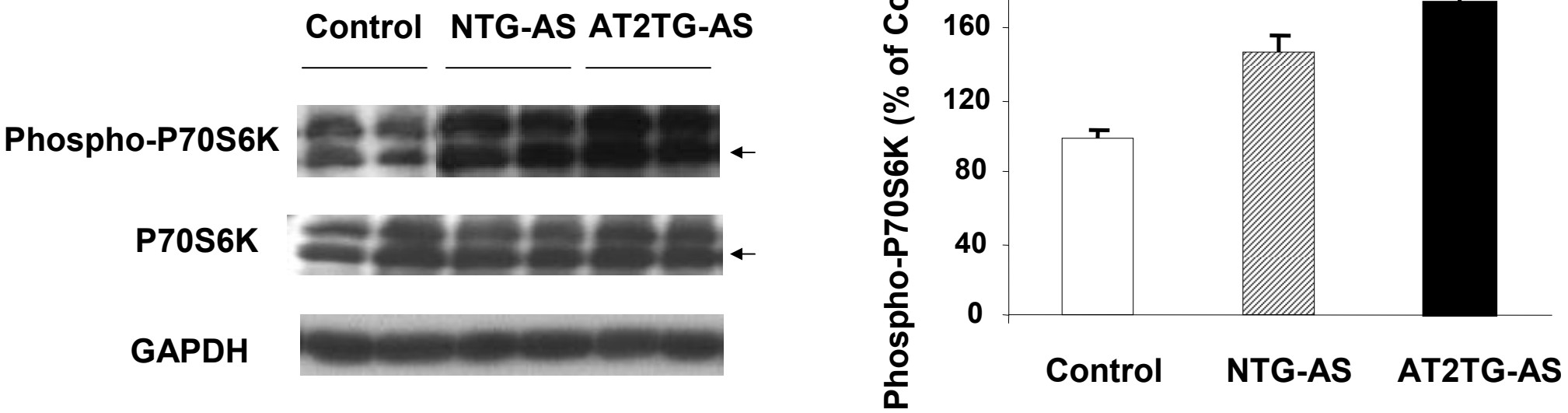


Figure 6